THE HAEMOCYANIN OF LIMULUS POLYPHEMUS.

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The question whether homologous proteins are identical in different animals is one of considerable general interest. It has attracted much attention, and much care has been devoted to it by Osborne[1]. Abderhalden and Schittenhelm[2] have also published upon it. The former compared vegetable proteins, the latter different caseins. Hæmocyanin offers a particularly favorable material for such a study, since, while it is supposed to have the same functions everywhere, it occurs in organisms less closely related than the different species of mammals or the different species of legumes. Henze[3] has made a very complete study of the hæmocyanin of the octopus (*Octopus vulgaris*). He has succeeded in crystalizing it by the original method of Hofmeister as well as by the Hopkins-Pinkus modification, and he has determined a good many of the amino-acids yielded on hydrolysis. We studied the hæmocyanin of *Limulus* and compared our results with those of Henze upon *Octopus*. *Limulus* offers exceptionally good material for a study of this kind, since in the early summer it is very abundant about Woods Hole, and a large female may yield as much as 400 cc. of blood.

The blood was obtained by making an incision in the back at the joint between the head and the abdominal piece. The yield

[1] Osborne and Clapp: *Amer. Journ. of Physiol.*, xx, p. 494; Osborne and Heyl: *Ibid.*, xxii, p. 423.

[2] *Zeitschr. f. physiol. Chem.*, xxvii, p. 458.

[3] M. Henze: Zur Kenntniss der Hæmocyanins, *Zeitschr. f. physiol. Chem.*, xxxiii, p. 370. Zur Kenntniss der Hæmocyanins, II. Mitteilung, *Ibid.*, xliii p. 290.

of blood may be increased by doubling up and straightening out the animal at this joint, like opening and closing a bellows, thus squeezing out nearly all of the blood. The blood was allowed to clot, and when the clot had contracted the serum was strained through a cloth. It was then placed in the ice-box for twelve to twenty-four hours in order that the floccules, which we have described in a previous paper[1] (and which Loeb[2] before us has shown simulate a second coagulation), might settle out. These were filtered off. They contain a most interesting protein, upon which we hope to report before long. From the clear serum the hæmocyanin was prepared in one of two ways:

(a) It was dialyzed until all the hæmocyanin was precipitated. The precipitate was then filtered off; dissolved in 5 per cent sodium chloride and fractionated with ammonium sulphate. We think this is not a very desirable method, for the hæmocyanin does not always precipitate completely. We do not quite understand the conditions for the complete precipitation of hæmocyanin by dialysis, but we are under the impression that too lengthy dialysis causes some of it to go into solution. Moreover, we suspect, as we will explain later, that some of the copper may be lost in the process.

(b) The serum was fractionated with ammonium sulphate. We believe this method to be preferable. As the serum was very alkaline it was neutralized carefully with very weak acetic acid, not stronger than .05 per cent, which must be added drop by drop, stirring constantly. If the acid is added too rapidly, or in excess, the hæmocyanin is decomposed, the protein part precipitating and more or less copper remaining in solution. At no time must the serum be allowed to become even faintly acid. It is best to cease adding acid when the reaction is still faintly alkaline. Under these conditions the first protein precipitation appeared when 3.3 parts of saturated ammonium sulphate were contained in a total volume of 10 cc. At this concentration the amount precipitated was slight. It was bluish, showing that it contained hæmocyanin. At a concentration of 4.5 cc. of ammonium sulphate in 10 cc. practically all the hæmocyanin was precipitated. Traces still came

[1] This *Journal*, v, p. 323.

[2] *Beitr. z. chem. Physiol. u. Pathol.*, v, p. 194.

down at a concentration of 4.7 cc. in 10 cc. Further addition caused no precipitation until a concentration of 5.5 cc. in 10 cc. was reached. This precipitate was not very voluminous. It was white, and therefore not hæmocyanin. A last, scanty precipitate was brought down by complete saturation with ammonium sulphate.

All in all, there is very little protein in the blood except hæmocyanin. Indeed we believe, though we have not yet demonstrated it as a fact, that all the protein present except the hæmocyanin is derived from the disintegrating cells of the clot. We base this view on the observation that the serum collected before the clot has begun to contract much is poorer in these proteins than that obtained after full contraction of the clot. We did not pursue this line of investigation further because it ought to be carried out on animals before spawning, when they are in the best condition. Our animals were used later, sometimes after having been kept in a floating car in the harbor several weeks.

We therefore precipitated the serum with 80 cc. of saturated ammonium sulphate solution for each 100 cc. of serum. The crude hæmocyanin thus obtained was filtered off, dissolved in water, and reprecipitated with ammonium sulphate. The necessary ammonium sulphate was added slowly until a concentration of about 3.4 to 3.5 in 10 cc. was attained. The precipitate thus obtained was filtered off and rejected. Then more ammonium sulphate solution was added to the filtrate until a saturation of about 4.4 cc. in 10 cc. was reached. The precipitate thus formed was filtered off and preserved. In this way the material precipitated at the lower and upper limits was rejected, the presumption being that contaminating material was thereby removed. There is of course no guaranty that by this procedure protein with the same precipitation limits as hæmocyanin is removed. That no such protein occurs in the serum we are not prepared to say. We have encountered no evidence of its existence. The hæmocyanin precipitated was redissolved and reprecipitated twice more in this fashion. After the first precipitation the limits of salt concentration were a little higher than they were in the original serum, possibly because the serum itself has about the concentration of salts found in sea-water. After the last precipitation the hæmocyanin was redissolved and precipitated with alcohol, under which it was kept for some time

to coagulate it thoroughly. The alcohol was then decanted off and the coagulum washed free from salt. There was of course the danger that the coagulum might retain traces of the sulphate. If, however, the alcohol were added slowly, with constant stirring, a very light flocculent precipitate was obtained which was easily washed. Moreover, only just enough alcohol to coagulate should be added, so as to keep as much of the sulphate in solution as possible. The sulphate may of course be dialzyed away before adding the alcohol, and thus this source of error may be avoided. However, we feared losing copper (cf. below) more than adsorbing salt. The material thus obtained was analyzed, after having been powdered and dried to constant weight *in vacuo* over sulphuric acid at a temperature of 70°. It gave the following figures:

0.2174 gm. substance Preparation I yielded 0.1405 gm. H_2O and 0.3891 gm. CO_2:

H = 7.18 per cent.
C = 48.80 per cent.

0.2094 gm. substance Preparation II yielded 0.1324 gm. H_2O and 0.3769 gm. CO_2:

H = 7.02 per cent
C = 49.09 per cent.

0.1950 gm. substance Preparation I yielded 27.30 cc. N at 18° and 766 mm.:

N = 16.30 per cent

0.2221 gm. substance Preparation II yielded 31.02 cc. N at 19° and 760.5 mm.:

N = 16.06 per cent

Sulphur was determined according to Folin. 0.4086 gm. substance Preparation I yielded 0.0465 gm. $BaSO_4$:

S = 1.56 per cent

Copper was determined by incinerating in porcelain, and exhausting the ash with nitric acid. A trace of material, probably silica, did not dissolve. The presence of silica in the clot has already been reported by us[1]. The acid solution was evaporated to dryness with a little H_2SO_4. The solution taken up in a little very dilute H_2SO_4 was filtered and the copper determined electrolytically.

0.5115 gm. substance Preparation I yielded 0.0015 gm.Cu:

Cu = 0.29 per cent.

For the check analysis a little more material was taken and a different method used. The material in this case was dried to

[1] *Loc. cit.*

constant weight in the oven at 105°. The ash was exhausted with nitric acid, the nitric replaced with hydrochloric acid, the copper precipitated as the sulphide, the latter collected on filter paper, washed, ignited, converted into the nitrate, and the latter into the oxide by ignition.

0.9960 gm. substance Preparation II yielded 0.0034 gm. CuO:

Cu = 0.0027 gm. = 0.27 per cent

The hæmocyanin obtained by Henze from the octopus had a considerably different composition. The following are his results and ours side by side:

	OCTOPUS. (Average) *per cent.*	LIMULUS. (Average) *per cent.*
C	53.66	48.94
H	7.33	7.10
N	16.09	16.18
S	0.86	1.56
Cu	0.38	0.28
O	21.68	25.94

It would seem from this comparison that the two substances are different. The greatest difference is in the sulphur content which is too great to be due to differences in the methods of determination used. The difference in the copper content is also condsiderable if we assume that our values are correct. We realize fully that to establish them we need more determinations with larger quantities of material. Perhaps it may be found that animals more sluggish than the octopus possess hæmocyanins of lower copper content, and therefore less active oxygen carriers. Certainly their blood as a whole usually contains less copper.[1] The difference in the carbon content of our preparation as compared with that of Henze is also great, and argues for the individuality of this substance.

It is, of course, possible that the difference in our figures is due to the fact that we worked with an impure substance. We do not regard this as probable, although we must admit that we have not the same guaranty of purity that Henze had, for we were quite unable to crystallize this substance as he did his. The Hofmeister method failed us, while in its Hopkins-Pinkus modifi-

[1] Cf. v. Fürth: *Vergleichende chemische Physiologie der niederen Tiere.*

cation it could not be used at all because of the great sensitiveness of this substance to acids. If to the solution we added a few drops of acetic acid, as Henze did, we got a heavy, curdy precipitate which could not be brought back into solution except by the use of alkali. We tried to obtain this substance in crystalline form, both from the fresh serum as well as from purified hæmocyanin. We are well aware our negative results do not prove that the substance is uncrystallizable. The conditions are not as favorable for its crystallization in *Limulus* as they seem to be in *Octopus*. It is so difficult to prevent larger quantities of the blood from clotting that we were compelled to work with the serum instead of centrifugated blood. The process of clotting as shown by Loeb[1] consists of an agglutination of cells, accompanied by their disintegration. This, as already indicated, we believe to be accompanied by the passage of material from the cells into the serum, which might well interfere with crystallization. Furthermore, Henze[2] states that crystallization goes on well only with fresh blood from healthy animals. While we used fresh blood, we had to lose some time in getting rid of the clot. Moreover, our animals, while apparently in good condition, had been collected and kept in a floating car in the harbor several weeks before they were used. Possibly with animals taken before spawning and used immediately the result might have been different. While these considerations may account for our inability to obtain crystals, we are inclined to believe that the real reason is that *Limulus* hæmocyanin is a globulin.

So much for our quantitative studies, which all point to the individuality of *Octopus* and *Limulus* hæmocyanin. Our qualitative studies offer more evidence for the same conclusion.

Henze states that his hæmocyanin could not be precipitated by dialysis, and questions the results of Halliburton, who reported that it could be so precipitated. Henze does not seem to have taken into consideration that there might be more than one kind of hæmocyanin in nature, and that both he and Halliburton might be right, each for the hæmocyanin with which he worked. As a matter of fact, *Limulus* hæmocyanin may be completely precipitated from serum by dialysis. Solutions of pure hæmocyanin are not as

[1] *Loc. cit.*

[2] *Loc. cit.*, p. 374.

easily completely precipitated, and occasionally behave in an erratic way that we have not yet explained to our own satisfaction, though we think it was probably resolution from too long disalysis. Always, however, the greater part was precipitated. Our hæmocyanin behaved like a globulin, Henze's like an albumen. Our hæmocyanin could be completely precipitated by magnesium sulphate, Henze's could not be. 4.7 cc. saturation with ammonium sulphate completely precipitates it. Henze's is only precipitated by complete saturation. In this respect, too, *Limulus* hæmocyanin behaves like a globulin, *Octopus* hæmocyanin like an albumen. By carbon dioxide our hæmocyanin was only incompletely precipitated, agreeing in this respect with Henze's. It gave the protein precipitation reactions, the xanthoproteic, the biuret, and Millon's reaction. It was precipitated by the salts of the heavy metals.

We are not certain how far the behavior of *Limulus* hæmocyanin toward acids differs from that of *Octopus* hæmocyanin, for we do not quite understand (no doubt our own fault) how the statements of Henze, on pp. 377 and 380 of his first paper, are to be reconciled with one another. We get the general impression from reading his papers that our substance was more sensitive to acid than his. Very slight traces of dilute acid, including acetic, precipitated it as white floccules poor in copper. Further addition of acid did not redissolve it. The copper is in exceedingly loose combination.[1] It may even be completely removed by dialysis if the water used be kept very faintly acid. The dialysis must be very protracted, extending over a number of days. If enough salt be present, the protein will not be precipitated. A cold storage room, kept just above zero, enabled us to carry out this experiment without bacterial action.

The possibility of losing copper in this way kept us from employing dialysis as a step in preparation. We have convinced ourselves that lengthy dialysis sometimes, not always, results in a loss of copper even when, apparently, the solution has remained

[1] One of us has already called attention to the ease with which copper is removed from *Limulus* hæmocyanin (C. L. Alsberg: Beiträge zur Kenntniss der Guajakreaktion. *Arch. f. exp. Path. u. Pharmak.*, Supplementband, "Schmiedeberg Festschrift," 1908, p. 39); and he has also suggested (*ibid.*, pp. 41-42) that different hæmocyanins may differ in their susceptibility to acid.

neutral. We spoiled considerable material before we realized this possibility. One preparation was dialyzed five days in the cold storage room at a temperature but slightly above zero. It gave us the following figures for copper:

2.7999 gm. substance Preparation III yielded 0.0040 gm. Cu (electrolytic method):

Cu = 0.143 per cent

To make certain that the figures were correct, a larger quantity of material was used, and the sulphide method employed:

II

16.434 gm. substance Preparation III yielded 0.0291 gm. CuO:

Cu = 0.138 per cent

Like Henze, we were unable to obtain any copper compound analogous to hæmatin. We regard our substance as a copper globulin compound, while he regards his as a copper albuminate. We believe they are probably two different proteins, though we are well aware that this can be settled finally only by a quantitative hydrolysis for which we lack at present sufficient material.

SUMMARY.

The hæmocyanin of *Limulus* differs from that of *Octopus* in percentage composition, in its precipitability by dialysis, by full saturation with magnesium sulphate and by half saturation with ammonium sulphate, in not having been crystallized, and perhaps in being more sensitive to acid. There are therefore different hæmocyanins, and perhaps this fact accounts for the discrepancies in the literature concerning the properties of this substance.

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